BINDING OF Leu^5-ENKEPHALIN AND Met^5-ENKEPHALIN TO A PARTICULATE FRACTION FROM RAT CEREBRUM

Jean-Claude MEUNIER and Christiane MOISAND
Laboratoire de Pharmacologie et de Toxicologie Fondamentales, Centre National de la Recherche Scientifique, 205 Route de Narbonne, 31078 Toulouse Cédex, France

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1. Introduction

Leucine-enkephalin and methionine-enkephalin are two pentapeptides with morphine like properties, naturally present in mammalian brain. They were first characterized by Hughes et al. [1] as H–TyrGlyGlyPheLeu–OH and H–TyrGlyGlyPheMet–OH respectively and found to behave as potent, although short lived opiates in a variety of pharmacological tests in vivo [2]. They do interact with the so-called opiate receptor (OpR) in vitro as evidenced by their ability to inhibit the binding to OpR of a variety of non-peptide opiate agonists and antagonists [3,4].

We have made use of radioactive enkephalins to demonstrate directly their interaction with the opiate receptor in vitro. It is shown in this report that both enkephalins bind with nearly equal affinities to one class of non-interacting sites in a particulate fraction from rat cerebrum. Binding of both enkephalins cannot be distinguished on the basis of its inhibition by non-peptide opiates such as levorphanol or naloxone, but is differentially affected by a variety of cations. These results are compared with those that have recently appeared in the litterature and which favor a multiplicity of enkephalin binding sites in similar preparations [2,5,6].

2. Materials and methods

2.1. *Preparation of the crude mitochondrial fraction*

Male Wistar rats, weighing 150–250 g were killed by decapitation. Their brains (minus cerebella) were quickly removed and processed at 0–4°C. Conditions are given for one cerebrum. The cerebrum was homogeneized in 10 ml 0.32 M sucrose–1 mM Tris–HCl, pH 7.4, with 10 strokes in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged (1000 × *g*; 5 min), the pellet (P1) washed once and the combined supernatants (post-nuclear fraction) centrifuged at 15 000 × *g* for 15 min. The resulting pellet (P2 or crude mitochondrial fraction CMF) was homogeneized in 10 ml 1 mM Tris–HCl pH 7.4 and spun as before (15 000 × *g*, 15 min), to yield the osmotically-shocked CMF, referred to as P2,osm. P2,osm (about 50 mg protein) was resuspended in a final vol. 5 ml Tris–HCl, 50 mM, pH 7.4.

2.2. *Binding of labelled enkephalins to P2,osm*

Binding of tritiated leucine-enkephalin ([^{3}H]LE) and tritiated methionine-enkephalin ([^{3}H]ME) to P_2,osm was studied by the method of Pert and Snyder [7] in Tris–HCl 50 mM pH 7.4 supplemented with 20 μM bacitracin to minimize peptide degradation [3]. Protein concentrations, estimated by the method of Lowry et al. [8], were in the range 0.5–1.5 mg/ml. Briefly: reaction mixtures (0.5 ml or 1.0 ml) were incubated, always in triplicate, for 12 min at 35°C, with and without levorphanol (0.1 μM or 1 μM). They were then chilled down to 0°C (melting ice-bath) and filtered under suction through Whatman GF/B glass-fiber disks. Unbound or loosely bound radioactivity was washed away with two 10 ml portions of Tris–HCl 50 mM, pH 7.4, at room temperature. Retained radioactivity was counted with InstaGel (Packard, 10 ml/filter) in a Intertechnique SL30 liquid scintillation counter.

2.3. *Chemicals*

[3.5-Tyrosyl-^{3}H]enkephalin (L-5-leucine), 45.6 Ci/mmol and [3.5-tyrosyl-^{3}H]enkephalin (L-5-methionine), 31.0 Ci/mmol, the Radiochemical Center, Amersham. Dextrorphan and levorphanol, Hoffman-La Roche. Naloxone, Endo laboratories.

3. Results

3.1. *Binding of [^{3}H]LE and [^{3}H]ME to P2,osm*

Binding of [^{3}H]LE (in the range 1–40 nM) and of [^{3}H]ME (in the range 0.6–64 nM) to their membrane-associated sites in P2,osm was studied in the absence (total binding) and in the presence (levorphanol insensitive binding) of 0.1 μM levorphanol, a potent opiate agonist. Levorphanol insensitive binding of the enkephalins increased linearly with increasing concentrations of free ligand (not shown) and was considered non-specific. On the other hand, levorphanol sensitive (total minus non-specific) binding of both enkephalins was saturable and followed a simple Langmuir adsorption isotherm. Scatchard representations of the data (fig.1a and 1b) yielded straight lines, indicating one single class of non-interacting binding sites for [^{3}H]LE (K_d = 3.4 X 10^{-9} M) and for [^{3}H]ME (K_d = 3.6 X 10^{-9} M). Plateau values for both enkephalins were nearly identical and of the order of 0.1 pmol/mg protein, a value that is in good agreement with those reported for other radioactive opiates in similar preparations [9].

3.2. *Inhibition of [^{3}H]LE and [^{3}H]ME binding by dextrorphan, levorphanol and naloxone*

Figure 2a shows that binding of [^{3}H]LE and [^{3}H]ME at their K_d was little if any inhibited by dextrorphan at concentrations up to 100 nM whereas it was maximally inhibited (60%) in the presence of 100 nM levorphanol. When normalized to the same control value, inhibition by levorphanol of the binding of both enkephalins followed the very same curve, half maximum inhibition being achieved at 5 nM. Assuming levorphanol and the peptides compete for the same site, then the dissociation constant for levorphanol was calculated to be 2.5 nM. Figure 2b shows that naloxone, an opiate antagonist, also inhibited [^{3}H]LE and [^{3}H]ME binding at their K_d. Normalized inhibition curves of both peptides binding were superimposable, half maximum inhibition (30%) being achieved at 10 nM naloxone, maximum inhibi-

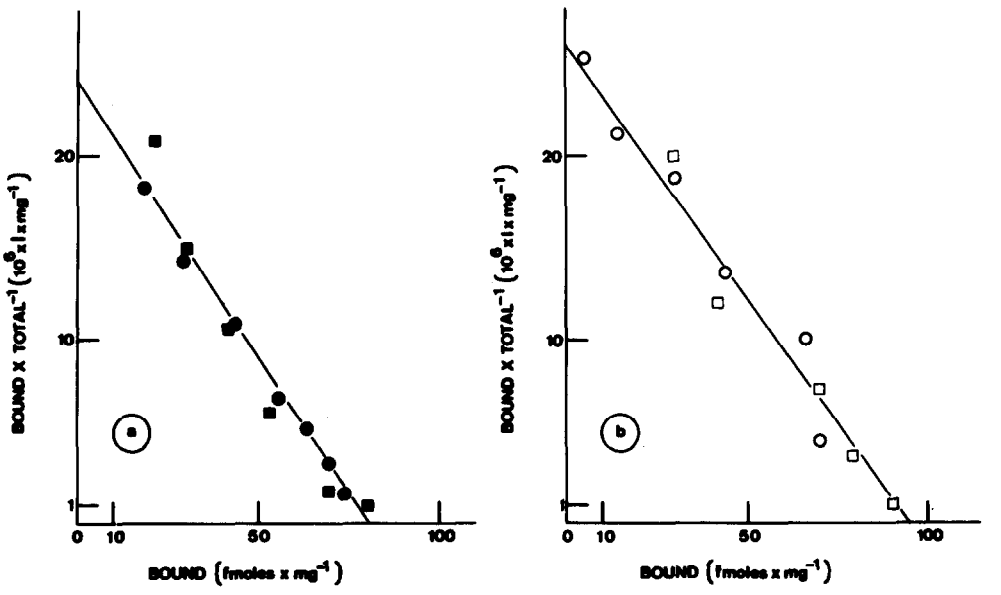

Fig.1. Scatchard representation of the levorphanol sensitive binding of (a) [^{3}H]LE (● ■), and (b) [^{3}H]ME (○ □) to a particulate fraction from rat cerebrum. Squares and circles represent separate experiments.

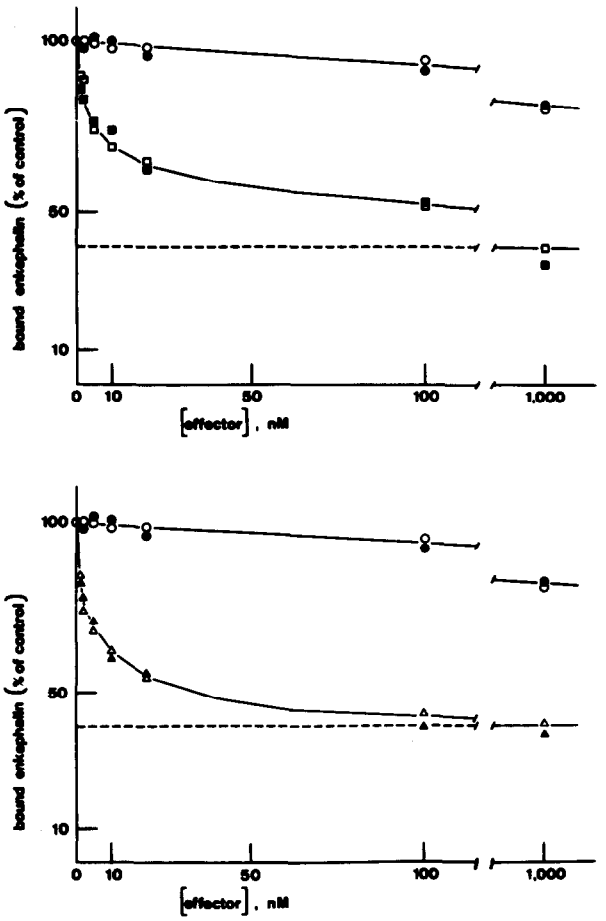

Fig.2. Effect of increasing concentrations of dextrorphan (●,○), levorphanol (▲,△) and naloxone (■,□) on [^{3}H]LE (closed symbols) and [^{3}H]ME (open symbols) binding at their K_d. Protein concentration of the incubation mixtures: 1.2 mg × ml^{-1}. Control values were: 3775 ± 127 cpm for [^{3}H]LE and 2581 ± 35 cpm for [^{3}H]ME.

tion (60%) at 1 μM naloxone. Although the affinities of levorphanol and naloxone for the opiate receptor are nearly identical [9], the later was found to be less potent an inhibitor of enkephalin binding than the former.

3.3. *Effect of cations on [^{3}H]LE and [^{3}H]ME binding*

The influence of various monovalent and divalent cations on the levorphanol sensitive binding of enkephalins to P_2,osm was studied. Figure 3a indicates that binding of [^{3}H]LE at its K_d was enhanced 40–60% over control by Mn^{2+} at concentrations in the range 0.25–10 mM and to a lesser extent (10–30%) by Mg^{2+} at similar concentrations. In contrast to this, Na^+ had an inhibitory effect: 50% inhibition at 20 mM, 80% at 100 mM. K^+ was much less effective: 5% inhibition at 20 mM, 35% at 100 mM. Binding of [^{3}H]ME (fig.3b) was found to be quite insensitive to Mn^{2+} and Mg^{2+} (up to 10 mM) and K^+ (up to 100 mM), but was inhibited by Na^+, although not as much, under similar conditions as [^{3}H]LE: 20% (versus 50%) at 20 mM, 60% (versus 80%) at 100 mM.

4. Discussion

The present study has shown that leucine- and methionine-enkephalin bind with a high affinity (K_d values of 3.4 nM and 3.6 nM, respectively) to one single class of non-interacting, membrane-bound sites in a particulate fraction of rat cerebrum. Recent reports, however, favor a multiplicity of enkephalin binding sites in similar preparations. According to Simantov and Snyder [3], Scatchard analysis of [^{3}H]ME binding to the opiate receptor reveals two distinct linear components with K_d-values of 0.64 nM and 2.6 nM. Morin et al. [5] describe binding of [^{3}H]ME to a particulate fraction from rat brain on the basis of two independent binding sites with K_d-values of 2.1 nM and 53 nM. Finally, Audigier et al. [6] using [^{3}H]LE present evidence for two saturable components with K_d-values of 2.7 nM and 30 nM, the low affinity component being insensitive to non-peptide opiates [10]. It must be pointed out that in contrast to the present study and that of Simantov and Snyder, Morin et al. and Audigier et al. have not selected as specific for enkephalins those sites that are levorphanol sensitive. Then their low affinity components might represent specific recognition sites not carried by the opiate receptor but by other membrane bound molecules involved in the synthesis, degradation, transport or secretion of the penta-peptides, a situation encountered before in other systems [11].

Binding of enkephalins to the opiate receptor (OpR) is not inhibited by 100 nM dextrorphan, but is maximally inhibited by 100 nM levorphanol. This result is consistent with the fact that dextrorphan is four orders of magnitude less potent than levorphanol

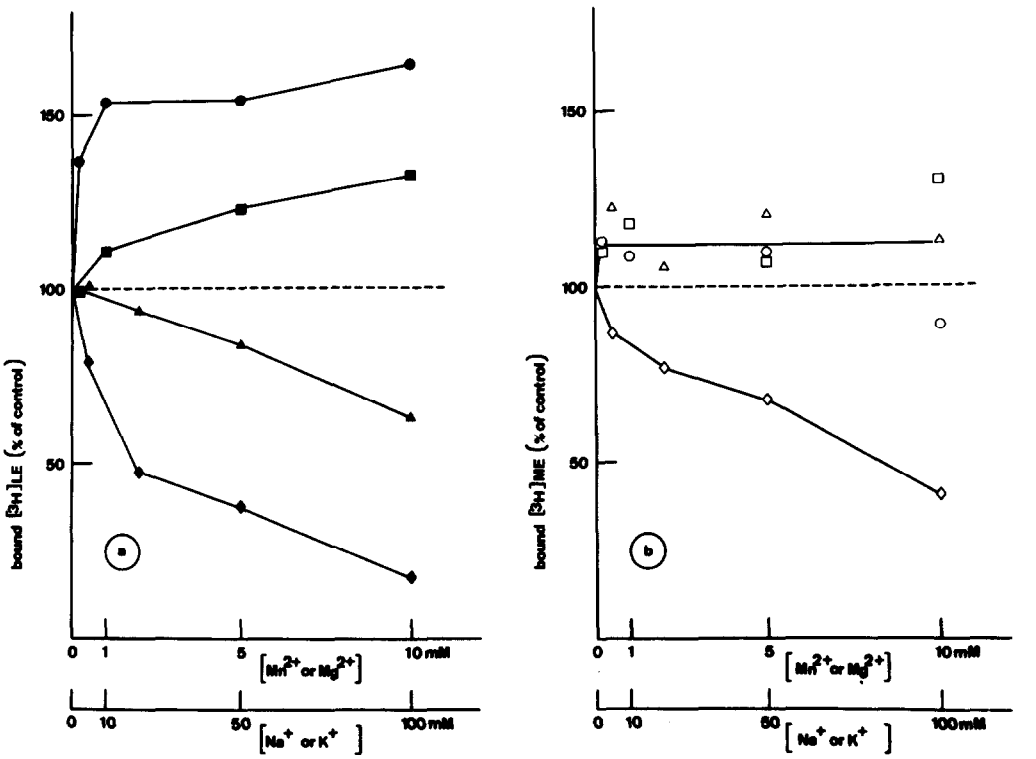

Fig.3. Effect of increasing concentrations of Mn^{2+} (●,○), Mg^{2+} (■,□), K^+ (▲,△) and Na^+ (◆,◇) on the levorphanol sensitive binding of [^{3}H]LE (a) (closed symbols) and [^{3}H]ME (b) (open symbols) at their K_d to a particular fraction from rat cerebrum. Protein concentrations of the incubation mixtures: 1.2 mg × ml^{-1}. Control values were: 1623 ± 104 cpm for [^{3}H]LE and 1523 ± 363 cpm for [^{3}H]ME.

in antagonizing binding of [^{3}H]naloxone to OpR [9]. Naloxone, an opiate antagonist, although it has an affinity nearly equal to that of levorphanol for OpR was less efficient than the later in inhibiting enkephalin binding. This is best explained by the fact that recognition sites for opiate agonists and antagonists are, at least partially, distinct: they can be discriminated upon treatment with enzymes [12] and protein-modifying agents [13].

Binding of enkephalin to OpR is differentially affected by a variety of cations. [^{3}H]LE-Binding at its K_d is enhanced by Mn^{2+} and Mg^{2+} and inhibited by Na^+ and K^+: this is typically an opiate agonist response [14,15]. On the other hand, [^{3}H]ME-binding at its K_d is quite insensitive to Mn^{2+}, Mg^{2+} and K^+ and inhibited by Na^+: methionine-enkephalin behaves like a mixed agonist–antagonist. These conclusions agree well with those of Simantov and Snyder [3]. Although we have not yet investigated this point, cations are likely to act upon the affinity of the pentapeptides for OpR as it has been shown to be the case for the opiate antagonists naltrexone [16] and naloxone [17].

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